III. CONCLUSION

21. From the foregoing analysis, I find that Spezyme Ethyl contains DNA having the nucleotide sequence set forth in Exhibit 3. This DNA encodes a protein, which has the amino acid sequence that is also depicted in Exh. 3.

22. I declare under penalty of perjury pursuant to the laws of the United States of America that the foregoing statements are true and correct.

Respectfully submitted,

Dated: 19. May 2005

Steen T. Jørgensen

Steen Troels Jørgensen

Attachments:

Exhibit 1: *Curriculum Vitae* of Steen Troels Jørgensen

Exhibit 2: Spezyme Ethyl DNA Sequence

Exhibit 3: P.L. Jørgensen *et al.*, "Cloning of a chromosomal α-amylase gene from *Bacillus stearothermophilus*" *FEMS Microbiology Letters* (1991) 77:271-276.

EXHIBIT 1

Steen Troels Jørgensen
Curriculum Vitae

Initials: **STJq**	**Full name:** **Steen Troels Jørgensen**
Date of birth: **8. June 1958**	**Reports to (initials):** **MDRa**
Year of graduation: **1985**	**University/college:** **University of Copenhagen**
Degree: **Cand. Scient**	**Joined NN in year:** **1985**

Job Experience:

Year:	Business unit/company:	Position:	Assignments/tasks:
1985	Bacterial Gene Technology, Novo Industri A/S.	Research Scientist	Gene technology as used in the construction of bacterial production strains for high-level expression of enzymes, and in the cloning and expression of novel enzyme coding genes.
1995	Bacterial Gene Technology, Enzyme Business, Novo Nordisk A/S	Principal Scientist (Science Manager)	As above.
2002	Bacterial Gene Technology, Novozymes A/S	Senior Science Manager	As above.

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EXHIBIT 2

FEMS Microbiology Letters 77 (1991) 271–276

ADONIS 037810979100051S

FEMSLE 04275

Cloning of a chromosomal α-amylase gene from *Bacillus stearothermophilus*

Per Linå Jørgensen, Gitte Bak Poulsen and Børge Diderichsen

Novo Nordisk, Bagsværd, Denmark

Received 22 August 1990
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Key words: Native plasmids; Heat stable amylases; Nucleotide sequence; Evolution; Cloning in *Bacillus subtilis*

1. SUMMARY

We have cloned and sequenced a gene for a heat-stable α-amylase from a natural isolate of *Bacillus stearothermophilus*. Previously, it had been shown that *B. stearothermophilus* amylase genes may be harboured on indigenous plasmids. We have found that our isolate harbours the amylase gene only on the chromosome and not on its indigenous plasmid.

2. INTRODUCTION

Amylases are exoenzymes, which are widely distributed in nature. All amylases, which have been sequenced so far, share significant homology suggesting an evolutionary relationship. The observation that an α-amylase gene from *B. stearothermophilus* is harboured on a natural plasmid [1] supports the notion that gene transfer can take place between related strains. In this paper we show that homologous amylase genes can reside either on a native plasmid or on the chromosome of different strains of *B. stearothermophilus*.

Correspondence to: B. Diderichsen, Novo Nordisk, DK-2880 Bagsvaerd, Denmark.

3. MATERIALS AND METHODS

3.1. Bacterial strains and plasmids

Escherichia coli K-12 MT102 is (*araD*139, Δ(*ara-leu*)7697, *lacX*74, *galU*, *galK*, *rpsL*, r^- m^+). *Bacillus subtilis* DN1885 (*amyE*, *amyR2*) is a derivative of *B. subtilis* 168 strain RUB200 [2]. *B. stearothermophilus* DN1792 and DN1793 were isolated from a household hot water system and classified by Deutsche Sammlung für Mikroorganismen. ATCC 31783 is a *B. stearothermophilus* from the American Type Culture Collection.

Plasmid pUN121 has been described [3]. Plasmid pDN2360 was constructed by ligation of *Pst*I fragments from pUN121 and plasmid pDN1380 [4] (Fig. 1). Plasmid pBB37 (Fig. 1) was constructed by inserting a *Cla*I fragment harbouring the gene for an α-amylase from *B. amyloliquefaciens* [5] into the *Cla*I site of plasmid pDN1620, which was derived from pDN1380 by insertion of an oligonucleotide linker in the *Eco*RI site.

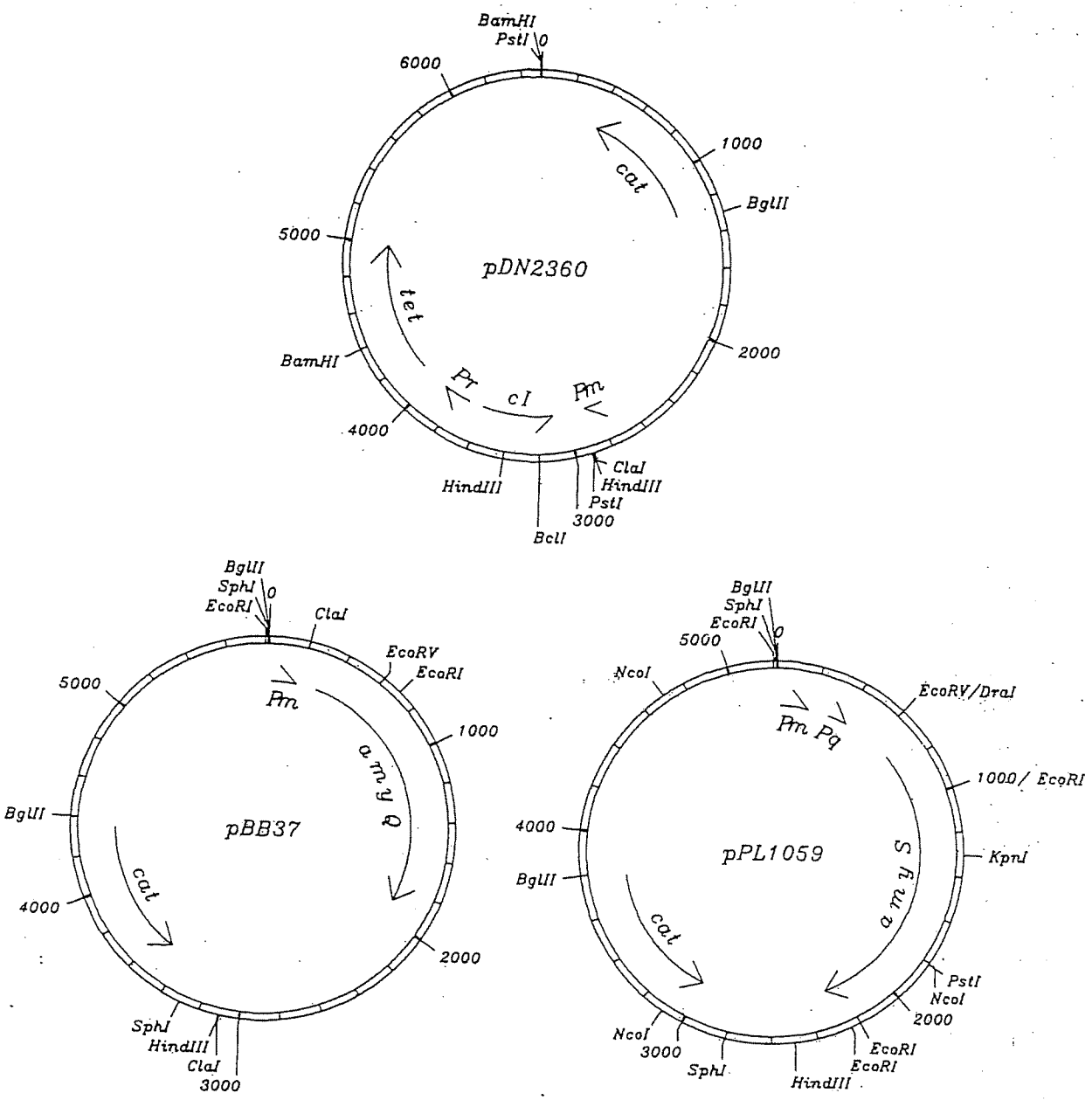

Fig. 1. Restriction maps of plasmids. pDN2360 replicates in both *E. coli* and in *B. subtilis*. pBB37 and pPL1059 replicate in *B. subtilis*. cI is the coliphage λ repressor gene. P_r is a promoter from λ. P_m is the promoter of the maltogenic amylase gene *amyM* [4]. *cat* is the chloramphenicol resistance conferring gene from plasmid pC194 of *Staphylococcus aureus*. *amyQ* is the amylase gene from *B. amyloliquefaciens* [5]. P_q is the promoter from the *amyQ* gene. *amyS* is the amylase gene from *B. stearothermophilus* described in this work.

3.2. Transformation and plasmid preparation

Competent cells, transformation, and preparation of recombinant plasmids were as described [4]. Transformants of *E. coli* were selected with 7 μg/ml tetracycline. Transformants of *B. subtilis* were selected with 6 μg/ml chloramphenicol.

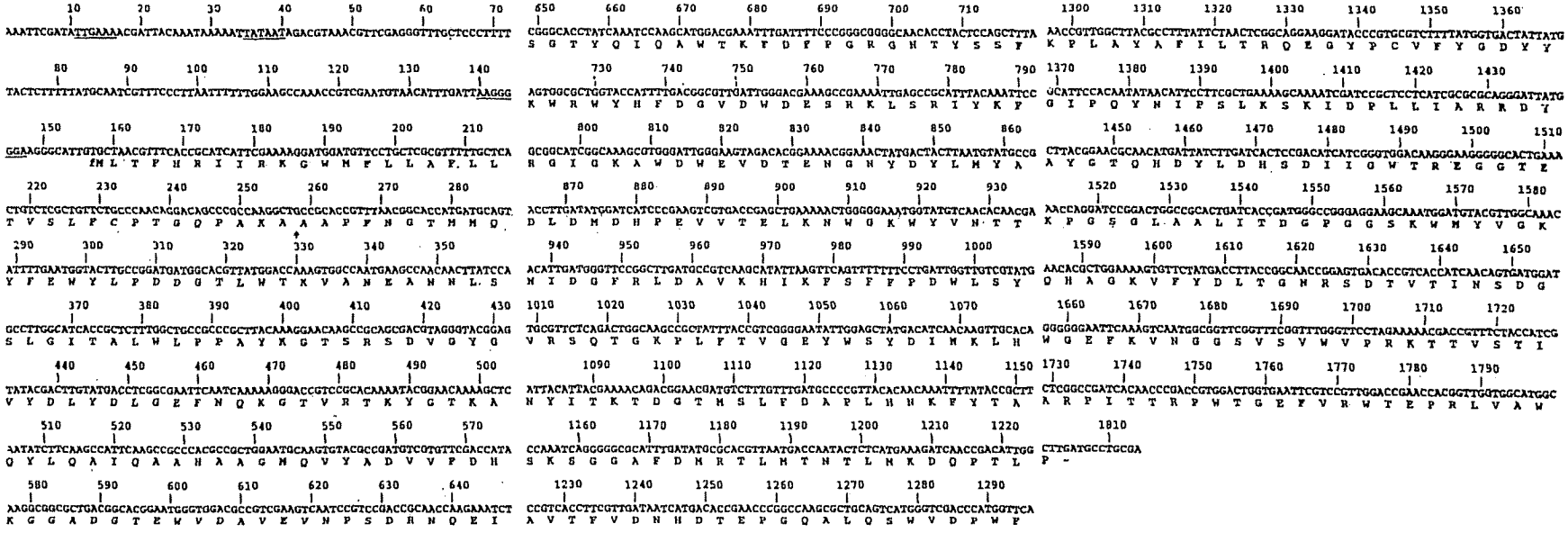

Fig. 2. DNA sequence of the *amyS* gene. Promoter regions −35 and −10 and a ribosome binding site have been underlined starting at bp 11, 35 and 140, respectively. An arrow shows the processing site after the 34 amino acid signal peptide.

Large plasmids were prepared by alkaline lysis according to ref. 6 with the exception that 50 mM glucose was added to the suspension buffer and that the samples were centrifuged for only 10 min before phenol extraction. Chromosomal DNA was prepared by phenol extraction [7].

3.3. DNA sequencing

DNA sequencing was performed directly on doublestranded plasmid templates using the Sequenase™ kit from United States Biochemical Corporation.

3.4. Southern analyses

Chromosomal and plasmid DNA was digested with restriction enzymes, fractionated on 0.7% agarose gels and blotted onto nitrocellulose filters. pPL1022 was labelled by nick translation using [^{32}P]dCTP (Amersham). The hybridization was according to Southern [8].

4. RESULTS

4.1. Cloning in E. coli

Genomic DNA from strain DN1792 was partially digested with *Sau*3A and fragments, of 3–10 kb were ligated with the *Bcl*I digested selection vector pUN121. *E. coli* MT102 was transformed with the ligation mixture selecting for tetracycline resistance on agar plates containing starch. Of 10000 transformants, two had a clear amylase positive phenotype. One of these contained a plasmid, pPL1011, with an insert of 8 kb. pPL1011 was partially digested with *Sau*3A and ligated with selection vector pDN2360 digested with *Bcl*I. Tetracycline resistant transformants of *E. coli* MT102 were screened for amylase expression and one positive transformant harbouring pPL1022 with an insert of 2.2 kb was kept for further study. The structural gene for the *B. stearothermophilus* amylase was called *amyS*.

4.2. Cloning in B. subtilis

A 2.0 kb *Dra*I-*Hin*dIII fragment of pPL1022 was ligated with the 3.3 kb fragment of *B. subtilis* plasmid pBB37 and transformed into *B. subtilis* DN1885 selecting for chloramphenicol resistance and screening for amylase activity. A positive transformant harboured the 5.3 kb plasmid pPL1059 (Fig. 1).

4.3. DNA sequence analysis

The sequence of the *amyS* gene of pPL1022 is shown in Fig. 2. Of the 1814 bp of this sequence, 33 bp (1.8%) differed from the sequence of Nakajima et al. [9] and 40 bp (2.2%) from the sequence of Gray et al. [10]. Comparing the amino acid sequence of the mature enzyme and the signal peptide with the above sequences showed 12 (2.2%) and 9 (1.6%) differences, respectively. Between the sequences of Nakajima et al. [9] and Gray et al. [10], there are 13 (2.4%) amino acid differences.

At the amino acid level, one of the three sequences differs from the two others at 17 positions of which 4 (11.8%) are in the signal peptide of 34 amino acids and the remaining 13 (2.5%) in the mature part of 515 amino acids.

4.4. Southern blotting analysis

To test the localization in *B. stearothermophilus* of the cloned amylase gene, plasmids were prepared from the *B. stearothermophilus* DN1792, DN1793 and ATCC 31783 [11]. DN1792 and DN1793 contained each one plasmid of 25 kb and ATCC 31783 contained three plasmids of 12, 15 and 26 kb as reported by Mielenz [1] (Fig. 3A). Using the cloned amylase gene as probe, hybridization was only seen with the 26 kb plasmid of ATCC 31783. The probe, however, clearly hybridized with the chromosomal DNA of DN1792 and DN1793 (Fig. 3B). Thus, *amyS* is located on the chromosome of DN1792 and DN1793 and on a native plasmid in ATCC 31783.

5. DISCUSSION

The *amyS* gene of a natural isolate of *B. stearothermophilus* has been cloned, sequenced and compared with genes from other *B. stearothermophilus* strains. The sequences of three independently isolated *amyS* genes (refs. 9, 10, and this work) show some variations in particular in the signal peptide regions.

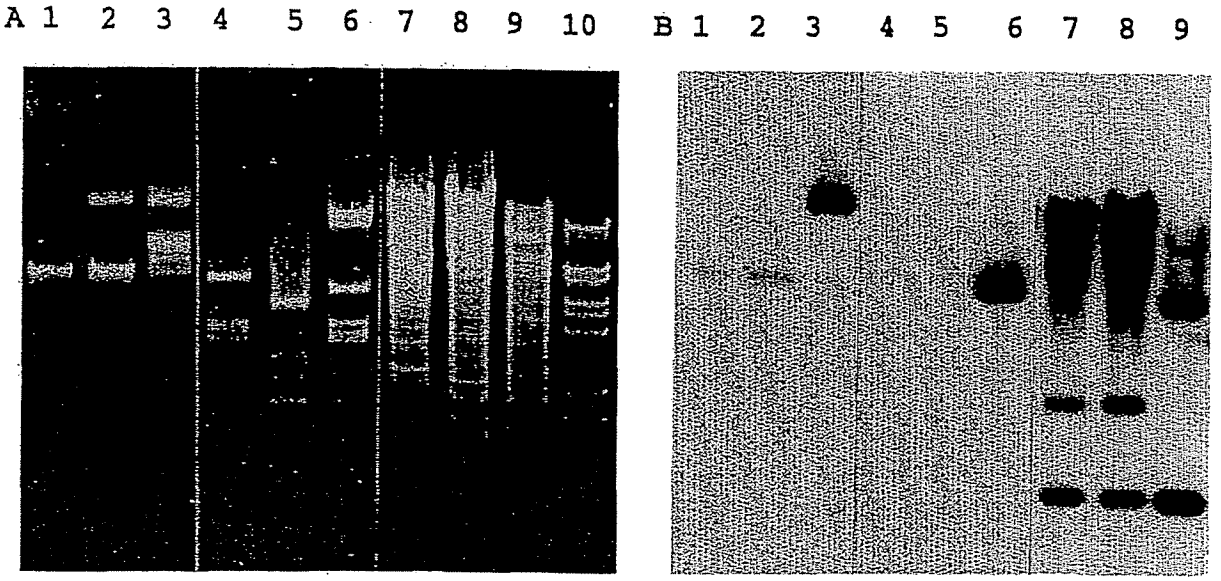

Fig. 3. Southern blotting analysis of *B. stearothermophilus* plasmids and chromosomal DNA. (A) 0.7% agarose gels stained by ethidium bromide. (B) The same gels as in A but hybridized with nicktranslated pPL1022. Lanes 1 and 4, plasmid from DN1792; lanes 2 and 5, plasmid from DN1793; lanes 3 and 6, plasmid from ATCC 31783; lanes 7–9, chromosomal DNA from DN1792, DN1793 and ATCC31783, respectively; lane 10, λ DNA digested by *Bst*E2. Lanes 1–3, uncut plasmids; Lanes 4–6, plasmids cut by *Hin*dIII; Lanes 7–9, chromosomal DNA cut by *Eco*RI.

Satoh et al. [12] have cloned two closely related amylase genes from what appear to be two phylogenetically very distant *B. stearothermophilus* strains. Nucleotide sequence analyses show that the regions flanking the amylase genes are completely different. Sen and Ariel [13] report on the location of amylase genes in two *B. stearothermophilus* strains. In one strain, the amylase gene is present both on two different indigenous plasmids and in multiple copies on the chromosome. In the other strain, multiple copies were found on the chromosome but no indigenous plasmid could be detected. We speculate that the multiple chromosomal genes in these two strains may be the result of plasmid integration. In this paper, it is shown that two other *B. stearothermophilus* strains harbour the amylase gene on the chromosome only, despite the presence of indigenous plasmids. It is thus likely that there is a mechanism in *B. stearothermophilus* which allows for migration of amylase genes from chromosome to plasmid or vice versa.

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